Lithium Toxicity and $\text{Na}^+(\text{Li}^+)/\text{H}^+$ Antiporter in *Escherichia coli*

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The lithium ion (Li^+) shows toxicity against *Escherichia coli* cells when present in a high concentration in the environment. Since Li^+ is extruded from cells via a $\text{Na}^+(\text{Li}^+)/\text{H}^+$ antiporter, this antiporter must be involved in the detoxification of Li^+ . Two $\text{Na}^+(\text{Li}^+)/\text{H}^+$ antiporters (NhaA system and NhaB system) are known to be present in *E. coli*. We investigated the properties of the antiporters and the participation of these systems in the detoxification of Li^+ using mutants lacking one of the antiporters, or lacking both of them.

Although the affinity for Li^+ of the two systems was almost the same, the $V_{\max}$ value for Li^+ transport of the NhaA system was about 12 times larger than that of the NhaB system.

Wild type cells were unable to grow in the presence of 0.7 M LiCl. Although a wild type cell and a mutant lacking the NhaB system grew in the presence of 0.6 M LiCl, a mutant lacking the NhaA system did not. This second mutant grew in the presence of 0.1 to 0.2 M LiCl. A mutant lacking both the NhaA and NhaB systems could not grow in the presence of 30 mM LiCl.

Keywords lithium ion toxicity; sodium ion (lithium ion)/hydrogen ion antiporter; inhibition; mutant; *E. coli*

The lithium ion (Li^+) inhibits the growth of bacterial cells and yeast.^{1,2)} We have investigated Li^+ toxicity in *Escherichia coli*.¹⁾ At low concentrations of Li^+ (10 mM), a difference in carbon sources affects the Li^+ toxicity. A stronger inhibitory effect of Li^+ on cell growth was observed when a glycolytic substrate such as glucose or fructose was the carbon source, rather than when an amino acid mixture or carboxylic acid such as lactate or succinate was the carbon source. Based on these observations, we found that one of the major targets of Li^+ inhibition in *E. coli* was pyruvate kinase.¹⁾ However, at higher concentrations of Li^+ (several hundred mM), the inhibitory effect of Li^+ on cell growth was not affected by the type of carbon source. Thus, it is very likely that (some) other biochemical pathway(s) is (are) also inhibited by Li^+ .

To obtain insight into Li^+ toxicity, it is important to investigate Li^+ transport in cells. In principle, Li^+ is impermeable to the cell membrane. Only two transport systems for Li^+ uptake are known in *E. coli*. One is the melibiose transport system,³⁾ and the other is the proline transport system I.⁴⁾ Li^+ is taken up by cells only when these transport systems and their substrates are present. At high concentrations, Li^+ may diffuse into cells very slowly. Three transport systems for Li^+ extrusion are known in *E. coli*: the NhaA system (Na^+/H^+ antiporter A), NhaB system (Na^+/H^+ antiporter B) and K^+/H^+ antiporter.^{5–8)} The first two systems extrude Na^+ or Li^+ in exchange for H^+ . The third system extrudes K^+ , Na^+ or Li^+ in exchange for H^+ . Since high concentrations of K^+ (200 to 300 mM) are present in *E. coli* cells,⁹⁾ extrusion of Li^+ via the third system usually does not take place. The respiratory chain (under aerobic conditions) and H^+ -translocating ATPase (under anaerobic conditions) extrude H^+ , and this establishes an electrochemical potential of H^+ across the membrane. This potential is the driving force for Na^+ or Li^+ extrusion via the NhaA system and the NhaB system. Thus, the respiratory chain is essential for Li^+ extrusion from *E. coli* cells under aerobic conditions.

We have shown previously that mutant cells which possess higher $\text{Na}^+(\text{Li}^+)/\text{H}^+$ antiporter activity were more resistant to Li^+ than wild type cells.¹⁰⁾ Also, it has been reported that cells harboring a plasmid carrying *nhaA* gene, which encodes for the NhaA system, were more resistant to Li^+ than wild type cells.⁵⁾ Thus, it is clear that the $\text{Na}^+(\text{Li}^+)/\text{H}^+$ antiporter is involved in the detoxification of Li^+ .

We have isolated or constructed mutants which were defective in the Li^+ extrusion system(s),^{11,12)} and investigated alterations in Li^+ -sensitivity in such mutants.

MATERIALS AND METHODS

Organism and Growth *E. coli* strain W3133-2,¹³⁾ a derivative of K12, was used as a wild type. Mutants were isolated or constructed using W3133-2 as a parent, and are listed in Table I.^{11,12)} Cells were grown in L-broth,¹⁴⁾ and when necessary NaCl was replaced with KCl; or in a modified Tanaka medium¹⁵⁾ supplemented with either 1% polypeptone, 40 mM lactate (K^+ salt) or 20 mM glucose at 37°C under aerobic conditions. When necessary, LiCl or KCl was added as indicated. Growth was monitored turbidimetrically at 650 nm.

Preparation of Membrane Vesicles Cells grown in L-broth were harvested at a late exponential phase of growth, washed with a buffer containing 10 mM Tris-HCl (pH 7.2), 140 mM choline chloride, 5 mM MgSO_4 , 6 mM 2-mercaptoethanol and 10% glycerol, and suspended in the same buffer. Cells were broken using a French press,

TABLE I. Strains of *E. coli* Used in This Study

Strain	Relevant genotype	Na^+/H^+ antiporter present
W3133-2	Wild	NhaA and NhaB
HIT-1	<i>nhaB</i> [−]	NhaA
HITΔAB ⁺	Δ <i>nhaA</i>	NhaB
HITΔAB [−]	Δ <i>nhaA</i> , <i>nhaB</i> [−]	None

and membrane vesicles were prepared as described previously.¹⁶⁾ Membrane vesicles were stored at -80°C until use.

$\text{Na}^+(\text{Li}^+)/\text{H}^+$ Antiporter Activity The $\text{Na}^+(\text{Li}^+)/\text{H}^+$ antiporter activity in membrane vesicles was measured using quinacrine as a fluorescent probe as described previously.¹¹⁾

Other Methods and Materials Protein contents were determined by the method of Lowry *et al.*¹⁷⁾ Reagents were obtained from commercial sources.

RESULTS

$\text{Na}^+(\text{Li}^+)/\text{H}^+$ Antiporter Activity in Mutants Cells of *E. coli* possess at least two practical $\text{Na}^+(\text{Li}^+)/\text{H}^+$ antiporters. To investigate the roles and properties of these two systems, we isolated or constructed mutants lacking one or both of these systems (Table I).^{11,12)}

The Na^+/H^+ antiport activity was measured with everted membrane vesicles prepared from wild type cells (possessing the NhaA system and NhaB system) or mutant cells (possessing the NhaA system or NhaB system, or none of these systems). Mutant (HIT ΔAB^-), which lacks both NhaA and NhaB, showed no Na^+/H^+ antiport activity under our experimental conditions (Fig. 1). Thus, we concluded that no major Na^+/H^+ antiporter other than the ones described in this paper are present in *E. coli* cells.

The Na^+/H^+ antiport activity in membrane vesicles prepared from mutant cells lacking the NhaB system (HIT-1) increased with pH. At pH 7.0 or 7.5, activity was not observed, but strong activity was observed at pH 8.5 or above. This pH dependency corresponds to a characteristic of the NhaA system.⁶⁾ Thus, the mutant HIT-1 really possesses only the NhaA system.

Membrane vesicles prepared from cells lacking the NhaA system (HIT ΔAB^+) showed Na^+/H^+ antiport activity which was not affected by pH; namely, the activity was almost the same from pH 7.0 to 9.0 (Fig. 1).

The wild type showed roughly the same activity as HIT-1 at pH 7.0 or 7.5, and very high activity, which was rough-

ly the sum of the two systems, at pH 8.5 or 9.0 (Fig. 1). These results are also consistent with the notion that there are only two major and practical Na^+/H^+ antiporters in *E. coli*.

We also measured the antiport activity of the mutants using Li^+ as a substrate. The activity of the NhaA system was again pH-dependent, the activity increased as pH increased, and that of the NhaB system was not affected by pH (data not shown). No Li^+/H^+ antiport activity was detected with membrane vesicles of HIT ΔAB^- (which lacks both NhaA and NhaB) at any pH tested in the presence of K^+ . Therefore, no detectable system which has the ability to act as a Li^+/H^+ antiport, other than the NhaA, NhaB and $\text{K}^+(\text{Na}^+, \text{Li}^+)/\text{H}^+$ antiporter, is present in *E. coli*.

It became possible to determine the kinetic parameters of the NhaA system and of the NhaB system, because our mutants possess only one of these systems. Table II shows the kinetic parameters of the two antiporters determined indirectly by the fluorescence quenching method. When Na^+ was the substrate, the K_m value for the NhaA system was 2- to 3-fold larger than that for the NhaB system. Thus, the NhaB system has a higher affinity for Na^+ . $V_{\max}$ values are almost the same in the two systems. On the other hand, when Li^+ was the substrate, the K_m values were almost the same in the two systems. However, the $V_{\max}$ values were very much different in the two systems. The NhaA system showed a $V_{\max}$ value more than 10 times larger than that of the NhaB system. This means that the NhaA system has much larger capacity for Li^+ extrusion than the NhaB system. Thus, it seems that the NhaA system is more important for the survival of cells in the

TABLE II. Kinetic Parameters in the NhaA System and NhaB System of *E. coli*

$\text{Na}^+(\text{Li}^+)/\text{H}^+$ antiporter	Substrate	K_m (mM)	$V_{\max}^a$
NhaA	Na^+	1.98	253
	Li^+	0.91	1000
NhaB	Na^+	0.73	305
	Li^+	0.63	78

a) Arbitrary units (% dequenching/s/mg protein).

Fig. 1. Effect of pH on Na^+/H^+ Antiporters in Membrane Vesicles of Wild Type Cells and Mutants

The antiporter activity in everted membrane vesicles prepared from wild type (possessing NhaA and NhaB) (○), HIT-1 (possessing NhaA) (●), HIT ΔAB^+ (possessing NhaB) (△) or HIT ΔAB^- (▲) was measured by a fluorescence quenching method. The assay mixture contained 10 mM Tricine-KOH (indicated pH), 140 mM KCl, 1 μM quinacrine and membrane vesicles (40 μg of protein). Quenching was initiated by the addition of lactate, and dequenching was initiated by the addition of 5 mM NaCl.

Fig. 2. Growth Inhibition by LiCl of Wild Type Cells

Wild type cells were grown in modified L-broth in which NaCl was replaced with either 0.7 M KCl (○) or 0.7 M LiCl (●) under aerobic conditions at 37°C .

Fig. 3. Effect of LiCl Concentration on Growth of Wild Type Cells and Mutants

Cells were grown in modified L-broth in which NaCl was replaced with 85 mM KCl under aerobic conditions at 37°C. LiCl was added to the medium at indicated concentrations. Symbols: ○, wild type (possessing NhaA and NhaB); ●, HIT-1 (possessing NhaA); △, HITΔAB⁺ (possessing NhaB); ▲, HITΔAB⁻.

Fig. 4. Effect of LiCl Concentration on Growth of HITΔAB⁻ Which Lacks both NhaA and NhaB

Cells were grown in modified L-broth in which NaCl was replaced with 85 mM KCl under aerobic conditions at 37°C. LiCl was added to the medium at 0 mM (○), 1 mM (●), 3 mM (△), 10 mM (▲) or 30 mM (□).

presence of high Li⁺ than the NhaB system.

Effect of Li⁺ on Growth Although wild type cells of *E. coli* were able to grow in the presence of 0.7 M KCl, they could not grow in the presence of the same concentration of LiCl (Fig. 2). However, cells grew in the presence of 0.6 M LiCl. Thus, concentrations of LiCl higher than this level are very toxic for wild type *E. coli* cells.

We tested the effect of concentrations of LiCl on the cell growth of wild types and mutants (Fig. 3). The wild type and a mutant lacking the NhaB grew in the presence of 0.6 M LiCl, but mutants lacking the NhaA, and lacking both NhaA and NhaB did not grow. Thus, the NhaA system is essential for cell growth at this LiCl concentration. At 0.3 M LiCl, a mutant lacking the NhaA system but possessing the NhaB system grew very slowly, but a mutant lacking both of the systems did not grow at all. A mutant lacking only the NhaA grew normally when the LiCl concentration was 0.1 M. Thus, the NhaB system alone is sufficient for cell survival when the LiCl concentration is around 0.1 M. Furthermore, 0.1 M LiCl is toxic enough for cells lacking both the NhaA and NhaB systems. Thirty mM LiCl was enough to prevent the cell growth of a mutant lacking the two systems, and 10 mM LiCl greatly reduced the growth of such cells (Fig. 4).

To gain insight into the target of the Li⁺ inhibition, we

TABLE III. Effect of LiCl Concentration on Growth

Strain	LiCl ^{b)}	Growth rate (doubling/h)			
		L-broth	Minimal medium ^{a)}		
			Polypeptone	Lactate	Glucose
W3133-2	0 mM	2.3	1.1	0.70	1.0
	30 mM	2.3	1.1	0.73	0.70
HITΔAB ⁻	0 mM	1.5	1.1	0.61	0.52
	3 mM	0.70	0.61	0.15	0.12
	10 mM	0.21	0.11	0.07	0.05
	30 mM	0.00	0.00	0.00	0.00

^{a)} Modified Tanaka medium supplemented with 1% polypeptone, 40 mM lactate (K⁺ salt), or 20 mM glucose, as indicated. ^{b)} LiCl was added to each medium at indicated concentrations.

tested the effect of a carbon source on the growth inhibition by Li⁺ (Table III). With wild type cells, 30 mM LiCl did not have any inhibitory effect when cells were grown in L-broth, or when cells were grown on polypeptone (amino acids mixture) or lactate. As reported previously, some inhibition of growth was observed when glucose was the carbon source.¹⁾ On the other hand, severe growth inhibition was observed with mutant cells lacking both the NhaA system and NhaB when 10 to 30 mM LiCl was used, regardless of the species of carbon sources. At 3 mM LiCl, however, more severe growth inhibition was observed when glucose or lactate was added as a carbon source than when an amino acid mixture was a carbon source.

DISCUSSION

It became clear that *E. coli* cells possess only two major and practical Na⁺(Li⁺)/H⁺ antiporters, NhaA and NhaB. Although we cannot exclude the possibility that other Na⁺(Li⁺)/H⁺ antiporters exist, such a system would possess only very weak activity if it exists. Among the two antiporters, the NhaA showed a larger capacity for Li⁺ transport than the NhaB. In fact, the NhaA alone was sufficient for cells to survive at high LiCl concentrations (up to 0.6 M). This implies that the NhaA system really extrudes great quantities of Li⁺ when Li⁺ is present in high concentrations. The NhaB system does not seem to

possess sufficient capability of extruding Li^+ under such conditions, because mutant cells possessing only the NhaB system were unable to grow at 0.4 to 0.6 M LiCl. However, the Li^+ -extruding capacity of the NhaB system was high enough when the concentrations of LiCl in the growth medium were up to 0.2 M.

Growth of mutant cells lacking both of the two antiporters was strongly inhibited by 10 to 30 mM LiCl. This inhibitory effect was stronger when glucose or lactate was a carbon source than when an amino acid mixture was the carbon source. Thus, one of the targets of Li^+ inhibition may be in a pathway of amino acid synthesis. Even in a rich medium such as L-broth, Li^+ (at 10 to 30 mM) inhibited cell growth severely. Thus, it is very likely that Li^+ also inhibits more important pathway(s) or function(s) of *E. coli* cells, other than pyruvate kinase and the amino acid synthesis system.

When *E. coli* cells are grown on glucose or lactate as a sole carbon source, how does Li^+ enter into cells? Two known Li^+ /substrate co-transporters (melibiose transporter and proline transporter I)^{3,4)} do not function under such conditions. Some influx of Li^+ through the $\text{Na}^+(\text{Li}^+)/\text{H}^+$ antiporter may take place when the Li^+ concentration is high. In addition, we cannot exclude the possibility that an unknown pathway(s) for Li^+ entry is (are) present in *E. coli* cells.

Recently, it has been reported that the defect in our mutant HIT-1 was not complemented with *nhaB* bearing plasmid.¹⁸⁾ This implies that the mutation in HIT-1 is not located in the *nhaB* gene, and that the mutation affects expression or activity of the NhaB system. An important point in our study, however, is that HIT-1 possesses only NhaA activity and no NhaB activity.

The mutants described in this paper would be useful for

cloning gene(s) encoding the $\text{Na}^+(\text{Li}^+)/\text{H}^+$ antiporter. In fact, we have cloned the $\text{Na}^+(\text{Li}^+)/\text{H}^+$ antiporter gene of *V. parahaemolyticus* using one of the mutants as a host strain (manuscript in preparation).

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